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# Pathological Effects of Repeated Concussive TBI in Mouse Models: Periventricular Damage and Ventriculomegaly

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**Pathological Effects of Repeated Concussive TBI in Mouse Models:  
Periventricular Damage and Ventriculomegaly**

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**BS Biological Sciences with Honors**

**BS Physiology & Neurobiology**

**Minor Molecular & Cell Biology**

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## **Abstract**

Repeated concussive traumatic brain injury (RcTBI) is the most prominent form of head injury affecting the brain, with an estimated 1.7 million Americans affected each year (Kuhn 2012). Neurologists have been concerned about the danger of repeated head impacts since the 1920's, but researchers have only begun to understand the long-term effects of **rcTBI** (McKee 2009). Although symptoms can be as mild as dizziness, current research suggests that multiple concussions can lead to a progressive degenerative brain disease known as chronic traumatic encephalopathy (CTE) (Luo 2008, McKee 2009, Kane 2013). Research on the brain is just beginning to scratch the surface of how to both facilitate the immediate recovery and treat the long-term physiological changes that result from concussive injury. In the Conover lab we have shown that a closed-skull weight drop mouse model of rcTBI is able to produce early CTE phenotypes in the periventricular septum and induce lateral ventricle expansion. Understanding the link between rcTBI and CTE in the mouse model can have practical future applications in developing therapeutics for the prevention, early diagnosis, and treatment of brain injury.

## Introduction

The consequences of repeated hits to the head are a concern for a variety of at-risk populations including contact-sport athletes, epileptics, and our military veterans exposed to explosive blast forces. Currently there are no treatments or pharmaceuticals available to combat CTE (Zhang 2015). The development of a clinically relevant mouse model for rcTBI will provide opportunities for the advancement of preventative and therapeutic procedures.

## Induction of Concussion

RcTBI results from multiple cranial impacts sustained by an individual in his or her lifetime. These impacts are closed-skull injuries that are predominantly caused by a high velocity impact that leads to rapid acceleration of the brain (Kuhn 2012). Immediate damage that results directly from the impact is considered a primary injury mechanism and is particularly attributable to shearing forces between different brain structures (Mierzwa 2015). Since cerebral structures vary in tissue density, acceleration rates are not constant throughout the brain upon impact. The shearing forces observed in response to varied acceleration rates within the tissue are found macroscopically around dense brain structures such as fiber tracts and ventricles, and also microscopically within neurons, glia, and blood vessels (Shitaka 2011). Primary injuries affect neurons, axons, and glia and lead to homeostatic imbalances and physiological changes that make up the secondary injury pathway (Kumar 2012). Secondary changes to the extracellular neurochemistry are involved in an important neuroinflammatory response that has been implicated in the development of degenerative diseases such as CTE (Kumar 2012).

## Chronic Traumatic Encephalopathy

Chronic traumatic encephalopathy (CTE) is a progressive neurodegenerative disease diagnosed in patients who have a history of multiple brain injuries (Stein 2014). There is often a

delayed onset of cognitive impairment that occurs after induction of rcTBI in CTE patients; the lag time of symptom onset can be years or even decades (Stein 2014). The early symptoms of CTE include irritability, aggression, depression, and short-term memory loss and are known to progress to dementia and Parkinson's-like conditions (McKee 2013). Although CTE is linked to a number of cognitive symptoms, none of them are distinctive enough to produce a reliable diagnosis; definitive diagnosis of CTE continues to require post-mortem tissue analysis. Post-mortem cerebral tissue can be marked by a number of cellular pathologies that include gliosis, neuronal loss, and the accumulation of hyperphosphorylated tau in addition to various structural changes in the brain such as cavum septum pellucidum and ventriculomegaly (McKee 2009). In the Conover lab, emphasis was placed on understanding the induction of ventriculomegaly and the early onset of damage to the periventricular septum in order to demonstrate a link between rcTBI and CTE in the mouse model.

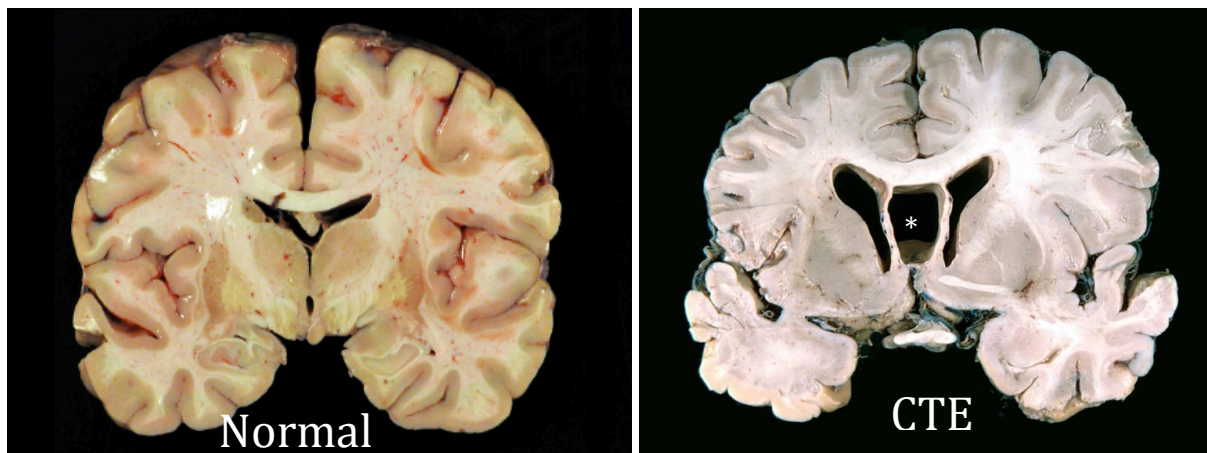


Figure 1. A coronal section of the adult human brain showing an intact periventricular septum and normal sized lateral ventricles (left). The brain of a patient with late stage Chronic Traumatic Encephalopathy (right). The asterisk marks the presence of a severe cavum septum pellucidum. The brain of the CTE patient also displays the expansion of the lateral ventricles associated with this neurodegenerative disease..

## Ventricle Enlargement

Ventriculomegaly is a condition of volumetric expansion of the lateral ventricles observed in cases of human neuronal degeneration and has been more recently described as a hallmark of CTE. The lateral ventricles, which make up the central portion of the ventricular system of the brain, are responsible for containing and facilitating passage of cerebral spinal fluid (CSF). Ventricles have an epithelial lining composed of an ependymal cell membrane that allows bidirectional transport between the CSF and the interstitial fluid, which plays a key role in maintaining ionic balance and protecting the brain from toxin accumulation.

Interestingly, the appearance of mild enlargement of the lateral ventricles is often observed at the early-onset Stage I of CTE, before any appreciable atrophy of neuronal tissue occurs. Consequently, it is understood that the progression of ventricle enlargement is not a

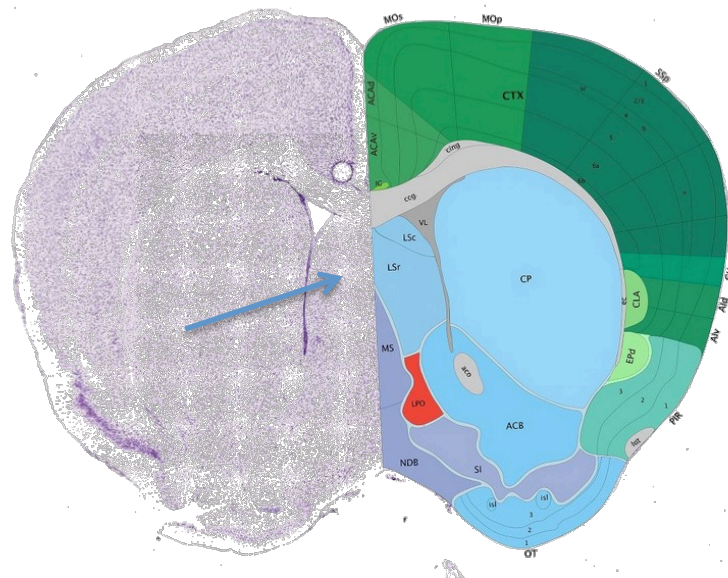


Figure 2. Coronal section of mouse brain depicting the anterior forebrain as both a low magnification DAPI image (left) and the digital atlas (right). The lateral septum (arrow) is located inferior to the corpus callosum and medial to the two lateral ventricles.

result of neurodegeneration but must have its own progressive mechanism (Stein 2014). The fact that lateral ventricles are at risk of experiencing fluid percussion waves during concussive head impacts is cited as a possible cause for focused damage leading to the ventricle expansion (McKee 2009). We attempt to unravel the mechanism behind ventriculomegaly by first showing that volumetric expansion of the lateral ventricle can be induced following rcTBI.

### **Damage to the Septum**

The second hallmark of CTE is the presence of severe cavum septum pellucidum (CSP) in rcTBI patients (Stein 2014). The septum plays an important role in the limbic system and contains neuronal projections, both cholinergic and GABAergic, which innervate numerous brain regions including the hippocampus, cortex, and amygdala. Experimental induction of non-impact injury to the septum has illustrated the susceptibility of this region to brain stress and injury (Park 2013). Specific alterations to neuronal activity of the septum, either through injury or interruption, have resulted in cognitive impairments similar to those observed in the clinical phenotype of CTE: memory loss, behavioral changes, anxiety (Chrobak 2006, Park 2013). Yet the majority of research done on repeated concussive TBI focuses on damage to the hippocampus, the cortex, and amygdala while overlooking the periventricular septum.

White matter tracts and fluid filled ventricles are especially vulnerable to rapid brain accelerations due their density differences as compared to neural tissue, and undergo increased strain and torsion with surrounding cerebral regions (Mierzwa 2015). The location of the periventricular septum inferior to the largest fiber tract in the brain, the corpus callosum, and flanked by the lateral ventricles (Figure 2, arrow) further underlines the septum's involvement in the development of CTE due to rcTBI. In this study we aim to observe cellular damage in the

septum after rcTBI through immunofluorescent histological analysis astrocytic and microglial pathological changes.

### **Histological Investigations of rcTBI**

We predict that reactive gliosis, a form of injury response by the astrocytes, plays a key role in the pathology of the rcTBI response and recovery especially in the septum. Astrocytes are the most prominent glial cell in the CNS and are responsible for maintaining the environment around neurons and neuronal synapses (Pekny 2014). Injury to the central nervous system causes astroglial cells to undergo both morphological and functional changes during activation. *In vivo* astrocyte activation is important in the neuroinflammatory response of neurodegenerative disorders and has a dual role in recovery (Pekny 2014). The changes in astrocytes include increased synthesis of an important intermediate filament, glial fibrillary acidic protein (GFAP), which enables structural growth of the astrocytic cytoskeleton and enhanced cellular signaling to other glial cells such as microglial macrophages. Both of these astrocytic changes provide astrocytes with the ability to envelop damaged lesions and isolate its toxic environment to assist in protecting surrounding healthy tissue. This neuroinflammatory protection is beneficial in post-acute trauma stages but can become inhibitory to CNS regeneration if it persists and is not resolved over time (Pekny 2014). The inhibition of regeneration by persistence of reactive gliosis may play a role in the development of CTE neurodegeneration. We are able to visualize and image reactive astrocytes by staining for the upregulation of GFAP with rat anti-GFAP antibody.

In addition, we predict that rcTBI will cause microglial changes as part of the neuroinflammatory response. Microglia are known to both aggregate in the regions of damage and begin their own morphological changes at injury lesions. Microglial macrophages act like



the immune system of the brain; expressed throughout CNS tissue, they are responsible for clearing cellular debris and releasing molecules to affect the inflammatory response. Activated microglia can be classified as one of two activated subtypes: M1, which help initiate the immune response by the release of pro-inflammatory cytokines and M2, which balance the response by releasing anti-inflammatory molecules (Wang 2013). It is believed that M1-activated microglia are critical in early protection around lesions caused by rcTBI (Kumar 2012). We are able to visualize microglia by staining against ionized calcium-binding adapter molecule 1 (IBA1), which is a cellular membrane protein upregulated by activated microglia.

While diagnostic capabilities are being explored using various advanced imaging technologies, including diffusion tensor imaging (DTI) and MRI, *in vivo* approaches still lack the resolution required to observe the cellular phenotype of CTE. Therefore post-mortem tissue analysis through immunofluorescent staining remains the only effective strategy to investigate the pathology of CTE and highlights the need for the development of a clinically relevant animal model to study its progression and discover possible therapeutics strategies.

### **RcTBI Mouse Model in the Conover Lab**

Currently, the Conover Lab is testing a clinically relevant mouse model of CTE in response to rcTBI through our collaboration with the Kuhn Lab at John D. Dingell VA Medical Center and Wayne State University School of Medicine, Detroit, MI. Our collaborators, affiliated with and supported by the Veterans Hospital, have developed a mouse model to study both sports- and military-related head injuries through a closed skull weight drop protocol (Kane 2012). The protocol is an alternative to many acute brain injury animal models, which do not properly mimic the acceleration and deceleration severity seen in concussive injuries and the

repetitive nature of rcTBI. Even in closed-skull rodent models of TBI, the head is often restrained and the animal is supported on a cushioned pad, in an attempt to limit the movement and forces to only those directly from the impact. Research has shown that fixed-head injury induction diminishes or completely removes the consequences of free head acceleration, including rotation, which can be the most destructive and come with the greatest risk of concussion (Kane 2012). The weight drop model developed by the Kuhn Lab removes any type of head restraint and allows the mouse free rotation as it falls to the cushioned bedding following head impact (Figure 4-b).

We utilize the Kuhn Lab's mouse model to research pathological progression of CTE phenotypes that occur in the periventricular regions of the lateral ventricles after rcTBI. The aim of my project is to (1) characterize the periventricular septum as a region particularly susceptible to CTE-patterned cellular damage and (2) analyze the ability of rcTBI to influence expansion of the lateral ventricles in a mouse model.

## **Materials and Methods**

### **Weight Drop Protocol**

Our collaborators at Wayne State University, under the approval of Wayne State University Institutional Animal Care and Use Committee, perform all the procedures involving the use of animals in this study.

The mice receive brief isoflurane anesthesia before being laid on the perforated aluminum foil stage. The head is aligned under the vertical guide tube that directs the 95 gram weight to impact the top of the mouse's cranium after a drop from 1 meter. After impact, the mouse is propelled through the foil and undergoes a 180° free rotation before landing supine on

the collecting sponge cushion below (Figure 4-c). The mice are moved to a carrying container where they quickly awake from anesthesia and right themselves. C57 and CD1 mice undergo five rounds of anesthesia and weight drop over a three-day period and are then either sacrificed immediately or are kept in normal living conditions until time of sacrifice (Figure 4-a). Control mice undergo a sham protocol that only involves treatment with isoflurane anesthesia with the same time line of five bouts over three days.

Sacrifice was performed by cardiac infusion with saline and 4% paraformaldehyde before the brains are extracted and perfused for 48 hours in PFA. Brains were rinsed in phosphate buffer solution (PBS), and shipped overnight to our lab at the University of Connecticut.

### **Tissue Processing and Immunohistochemistry**

The brains were mounted on a vibratome (VT-1000S; Leica) in agarose gel and sectioned to produce a sequence of 50 $\mu$ m thick coronal slices (Figure 5-a). The coronal tissue sections from 1.98 anterior to 2.54 posterior to Bregma are blocked in 10% horse serum (Invitrogen) in PBS/0.1% Triton X-100 for one hour. For cellular damage analysis under fluorescent microscope tissue sections along the periventricular region were immunostained with rat anti-GFAP (Abcam), goat anti-AQP4, mouse anti-s100 $\beta$ , rabbit anti-IBA1 (Wako) antibodies (Figure 5-b). Glial fibrillary acidic protein (GFAP) is used to identify areas of surface gliosis surrounding the lateral ventricles where the ependymal lining has been compromised (Shook 2013). The s100 $\beta$  antibody illuminates the dense ependymal monolayer of cells that exists surrounding cranial ventricles (Shook 2013) and allows for accurate outline tracing during ventricular reconstruction.

After coronal tissue was incubated for 24 hours at 4°C in primary antibodies it was rinsed with PBS three times. Alexa Fluor dye-conjugated secondary antibodies (Invitrogen) were applied for one hour at room temperature before PBS rinse. Tissue was then treated DAPI nuclear stain for 10 minutes followed by three final PBS rinses. The immunostained sections are then mounted sequentially; starting with the most anterior, on microscope slides and coverslipped with aqua-poly mount (Polysciences).

### **Imaging of cellular structures**

Fluorescent multi-channel images were obtained after immunohistochemical treatment of coronal tissue sections. Imaging was performed at various magnifications with Carl Zeiss Axio Imager M2 Microscope with Apotome (Carl Zeiss). Exposure and light settings were controlled throughout imaging.

### Three-Dimensional Ventricle Reconstruction

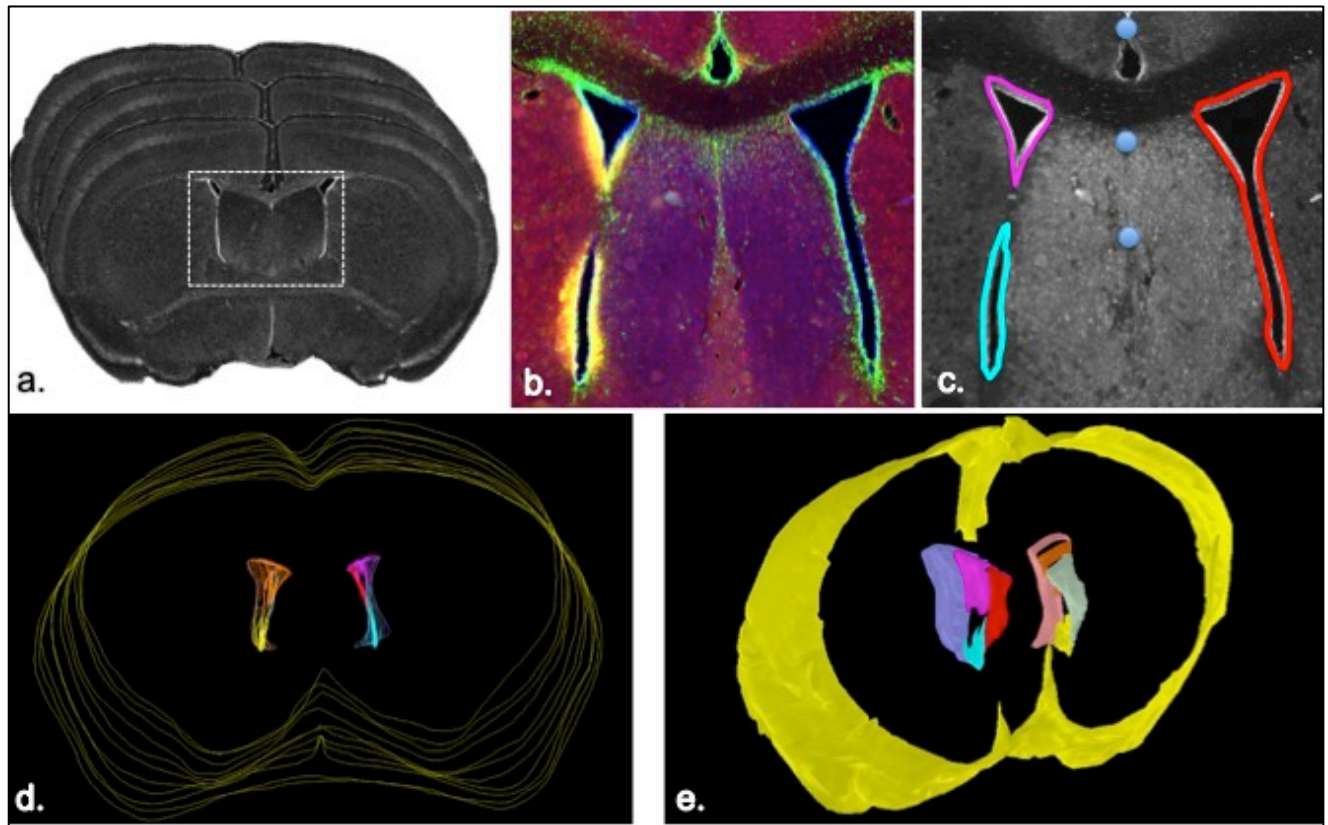


Figure 3. a. Coronal sections of mouse brain, periventricular region of interest highlighted with dotted box. b. Immunofluorescent staining on periventricular coronal tissue section. c. StereoInvestigator contour tracings overlaid on ventricle ependymal visualized with S100 $\beta$  staining. d. Neurolucida Explorer compilation of two-dimensional ventricle contour traces prepared for reconstruction. e. Three-dimensional reconstruction of lateral ventricles displayed in Neurolucida Explorer.

To begin ventricle reconstructions the anterior boundary of the lateral ventricles must be located with a strong S100 $\beta$  signal. StereoInvestigator in conjunction with our Zeiss Axio Imager M2 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) is used to develop digital contours tracing the lateral ventricles (Figure 3-c). Unique contour types are assigned for each region (anterior, upper and lower stenosis, posterior) of the ventricles to allow specific regional analysis of the ventricular volume. Subsequently point markers are placed on cerebral structures including the midline to assist in faithful alignment of contours for reconstruction. In addition a contour trace of the full brain is completed on every fourth coronal

section to allow for full brain volume analysis. Each coronal section is traced, marked, and then saved as an individual data file. Neurolucida Explorer is used to compile the two-dimensional contour traces by overlaying and assigning z-axis depth values to each contour section. The depth values are assigned in 50 $\mu$ m increments to match the thickness of the coronal tissue slices (Figure 3-d). The digital ventricle contours are linked in space from their most anterior point caudally to the presence of the third ventricle. Three-dimensional models of the lateral ventricles are rendered with Neurolucida Explorer (Figure 3-e). The 3-D rendering models provide volume data of the ventricles and whole brain outline for expansion analysis compared to control specimen.

### **Quantification Techniques with ImageJ**

The degree of upregulation of GFAP was quantified to support observable findings in the immunofluorescent imaging. We performed preliminary above-threshold quantification of the GFAP in five distinct regions on the periventricular coronal section. After immunofluorescent imaging a green channel GFAP color split was generated in ImageJ. The image was then adjusted to only display pixels above the median threshold value. The step was crucial to remove residual background illumination in the images and instead only display the GFAP expression from astrocytes. Each quantification region was measured out to be square with an area of 4000 $\mu$ m<sup>2</sup> and placed over its specific region of interest. The area within the boundaries of the square was quantified to determine the area of above-threshold pixels, which is the area of GFAP expression. The quantification was completed on eight C57 control brains and the four rcTBI four-week post injury mice.

Preliminary quantification of lateral ventricle intrusion into the septum was also performed using ImageJ software. The distance between the dorsal-medial edges of the lateral ventricles was measured in micrometers using a straight-line measurement tool. Distance values were collected three times for each individual and averaged. Eight C57 controls and the four-week post C57 hit brains were sized to compare lateral ventricle separation.

### Statistical Analysis

Experimental values were compared to controls using a two-tailed unpaired T-test. The populations were considered significantly different with a p-value  $< 0.05$ .

## Results

### Weight Drop Protocol

Adult mice, aged seven to eight weeks, are randomly assigned as either anesthesia controls or test group subjects. The test group mice receive five concussive weight drop hits over a three-day period under light

anesthesia (Figure 4.a). The anesthesia controls undergo the same exposure to isoflurane and are laid on the apparatus but do not sustain a weight drop hit. After the conclusion of the impacts the mice are randomly distributed into

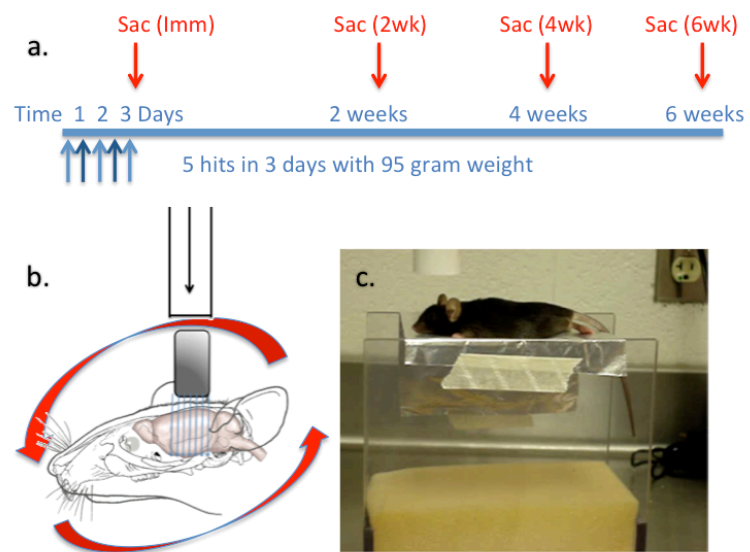


Figure 4. Weight drop protocol of mouse model developed by Kuhn Lab, Wayne State University. a. Weight drop timeline. b. Location of weight drop and depiction of free rotation of the mouse head. c. Mouse laying on perforated fine foil above collecting cushion and underneath vertical guide tube of rcTBI apparatus.

cohorts and sacrificed either immediately or at one of the later time points: two weeks, four weeks, six weeks, three months. Brains are removed, fixed in paraformaldehyde for two days, and shipped to our lab.

### What is the periventricular astrocytic response to rcTBI in the mouse model?

In order to examine transformation of astrocytes in response to rcTBI, we immunostained coronally sectioned neuronal tissue in the periventricular region with anti-GFAP fluorescent antibodies.. Fluorescently stained tissue was imaged at 10x magnification at the regions of interest for comparison of the cellular response between hit mice and age-matched controls.

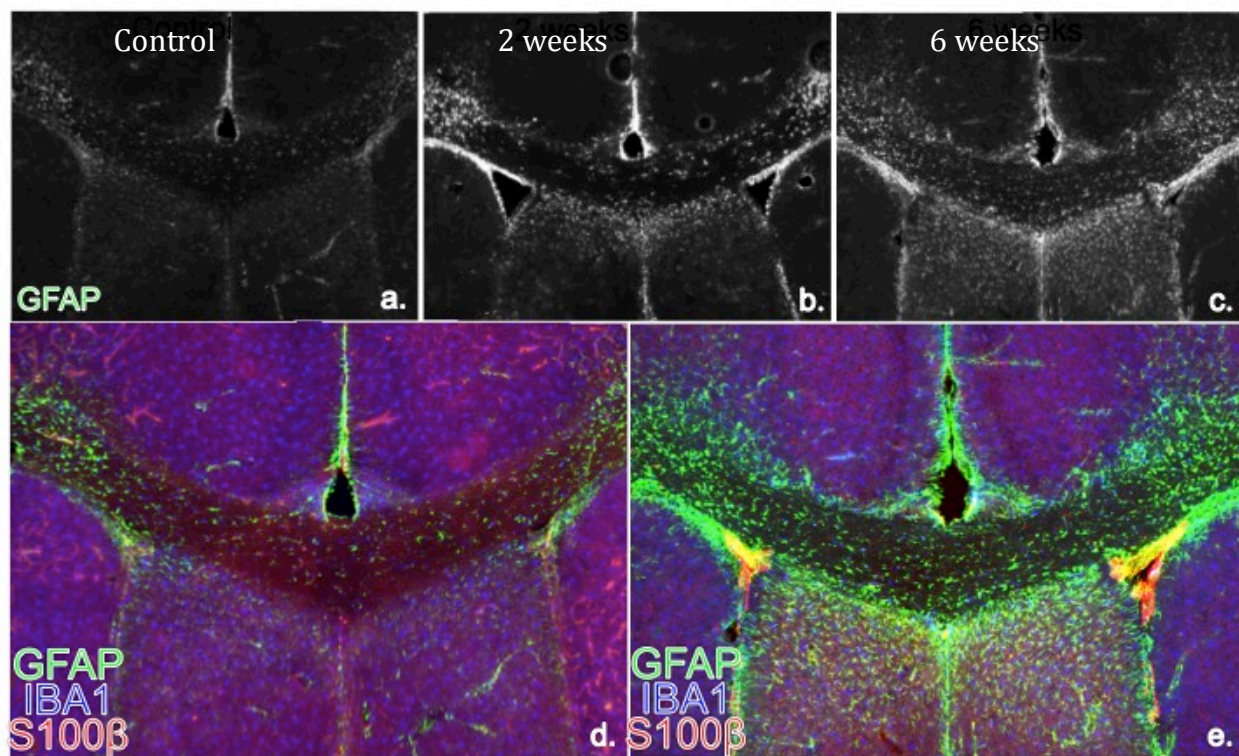


Figure 5. GFAP expression in CD1 mouse response to rcTBI. a. Baseline GFAP staining in control mouse. b. Increased GFAP observed in two weeks post rcTBI mouse especially around lateral ventricles and within septum along corpus callosum. c. Increased GFAP observed six weeks post rcTBI again depicts increased GFAP expression at lateral ventricles and in septum. d. Multichannel image of (a.)



showing baseline levels of GFAP, IBA1, and S100 $\beta$  staining in a control subject. e. Multichannel image of (c.) displaying increase in GFAP expression around lateral ventricles and corpus callosum in addition to IBA1 and S100 $\beta$  staining.

As expected, we found patterned, base-line GFAP fluorescence in the control tissue and observed that in control mice GFAP-expressing astrocytes are found sparingly in and around the medial corpus callosum, moderately along the lateral corpus callosum, and to a limited extent in the septum (Figure 5-a, 5-d). Our rcTBI model does not produce cortical damage in the mice and GFAP expression remains at very low, baseline levels in the cortex of hit mice (Figure 5-e, Figure 6-d) similar to the cortical GFAP found in controls. In the mice that underwent the rcTBI protocol we observed notable upregulation of GFAP in regions surrounding the lateral ventricle and corpus callosum (Figure 5-b, c, e and Figure 6-d).

Our hit to control comparisons in the periventricular region revealed a strong focal upregulation of GFAP in the superior region of the septum, along the boundary of the corpus callosum (Figure 5-d, 5-e).

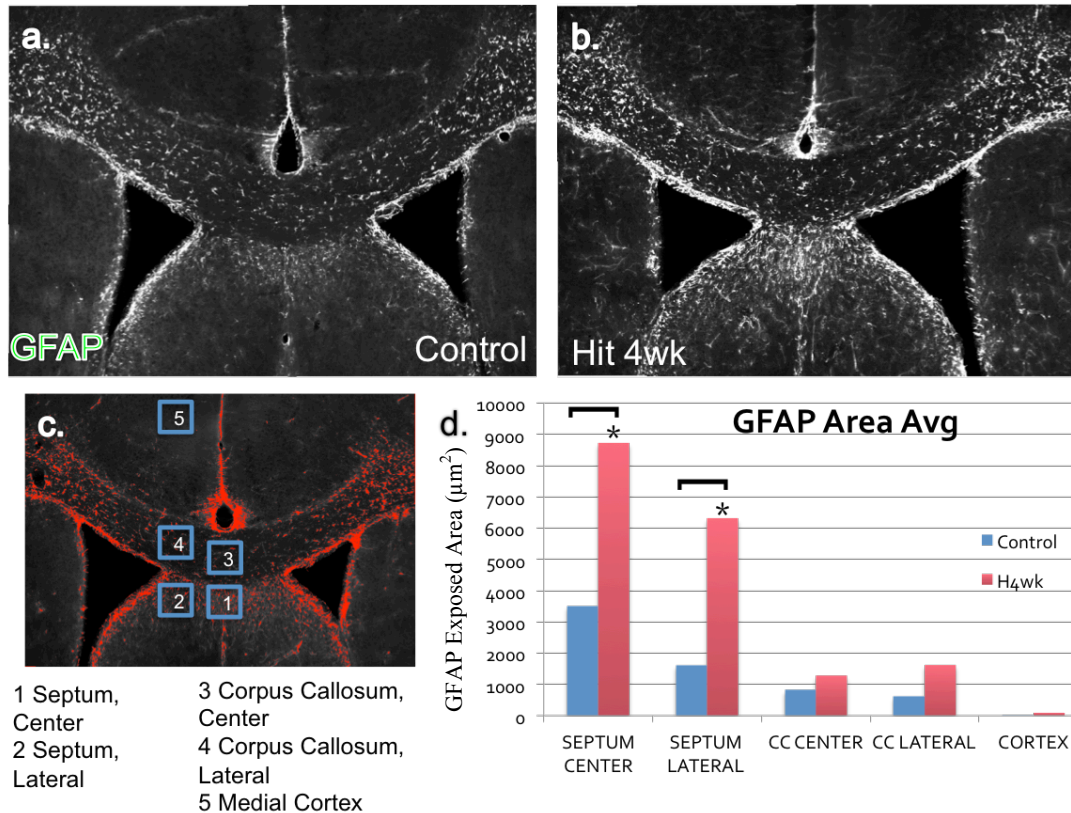


Figure 6. Above-threshold quantification of GFAP upregulation in C57 mice exposed to rcTBI. a. Baseline GFAP expression in control mouse. b. GFAP upregulation observed four weeks post injury, especially in inferior to corpus callosum in the superior region of the septum. c. Location of GFAP quantifications boxed and numbered. d. Significantly increased above-threshold GFAP area in septal regions in hit brains four weeks post hit compared to age matched controls.

The extent of periventricular GFAP upregulation in response to rcTBI was investigated with above-threshold pixel area analysis of fluorescent images. Quantification using ImageJ software was completed on C57 mice four weeks after exposure to repeated concussive impacts (n=4) and compared to age matched isoflurane controls (n=8). Five regions of the anteroventral cerebral tissue were selected for analysis: Midline septum, septum near lateral ventricle, midline corpus callosum, lateral corpus callosum, and medial cerebral cortex (Figure 7-c). GFAP expression was observed to be elevated in all regions of evaluation, except in the cortex where no substantial GFAP expression was observed in either control or hit brains (Figure 7-d). Significant increases of astrocytic GFAP were discovered in periventricular septum both at the midline and adjacent to

lateral ventricle,  $p\text{-value} < 0.05$  (Figure 7-d). Above-threshold GFAP quantification was only performed at a single time point after rcTBI and further analysis will be completed to gain better understanding of the progression of the astrocytic response.

### **Is there a localized microglial response to repeated concussive injury?**

Anti-IBA1 immunofluorescent staining was applied to coronal tissue and imaged to allow examination of the microglial response to repeated concussive injury. The control mice brains displayed evenly distributed microglia in their inactive, quiescent stage in all regions of the tissue at 10x magnification (Figure 7-a). At the immediate time point in the weight-drop exposed mice a strong upregulation of IBA1 was observed in the septum, inferior to the corpus callosum near the midline under 10x magnification (Figure 7-b). The septum revealed an aggregation of microglia cells in the anterior septum of the hit brain (Figure 7-d) compared to controls (Figure 7-c) at 20x magnification.

Morphological changes of individual microglial macrophages were analyzed with 40x magnification imaging of the septum (Figure 7 e-f). The control brains show quiescent, ramified microglia with a small cell bodies and spindly processes (Figure 7-e). Septal microglia in the rcTBI brains displayed enlarged cell bodies with reduced processes in the characteristic amoeboid shape of activated microglia in response to injury (Figure 7-f).

To determine the specificity of microglial changes to the septum, corresponding regions inferior to the corpus callosum within the striatum at the immediate time point were imaged. The strong microglial activation found in the septum of mice at the immediate time point after exposure to rcTBI was not observed in the striatum, which lies outside the boundaries of the lateral ventricles (image not shown). No such aggregation of activated microglia was found

along the corpus callosum in the striatum. Microglia of the striatum inferior to the corpus callosum resembled the ramified glia in the control mouse and did not depict the activated morphology seen in the septum (image not shown). All notable IBA1 upregulation was confined specifically to the lateral septum in the periventricular region.

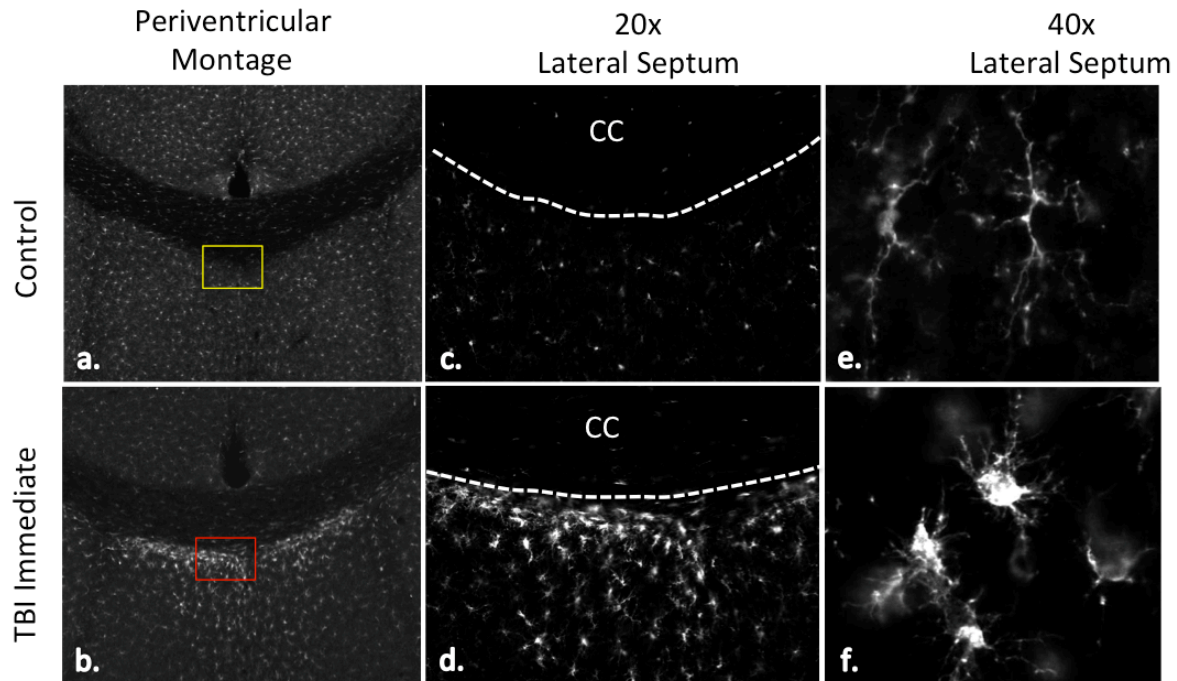


Figure 7. Microglial activation in CD1 lateral septum after rcTBI. a. Control periventricular coronal section with baseline IBA1 expression . b. Immediate time point after rcTBI displays upregulated IBA1 expression in septal region below corpus callosum; 20x magnification image location highlighted. c. 20x Magnification of control lateral septum inferior to corpus callosum (CC). d. Magnification of lateral septum inferior to CC immediately after rcTBI, increased expression of IBA1 and microglial aggregation. e. Quiescent microglia in lateral septum of control at 40x magnification. f. Activated ‘amoeboid’ microglia in lateral septum of immediate hit at 40x magnification.

The absence of cortical damage in our mouse model was again supported by the absence of activated microglia and the lack of IBA1 upregulation in the cortex.

### Ventricular invasion into the periventricular septum

Investigation into the structural changes of the septum was conducted at a single time point through quantified measurements of lateral ventricle separation along the corpus callosum. ImageJ software was utilized to measure distances along the corpus callosum between lateral ventricles at 10x magnification. Compared to age matched controls (Figure 8-b), mice sacrificed four weeks after the rcTBI protocol displayed a significant decrease in septum width between the lateral ventricles four weeks after injury (Figure 8-c). The average medial separation of the lateral ventricles in the hit brains at the four week time point (n=4) was significantly diminished compared to the control group (n=8) (Figure 8-a). At this point only C57 mice from the four-week time point were analyzed and compared to controls but future work will increase the number time points examined.

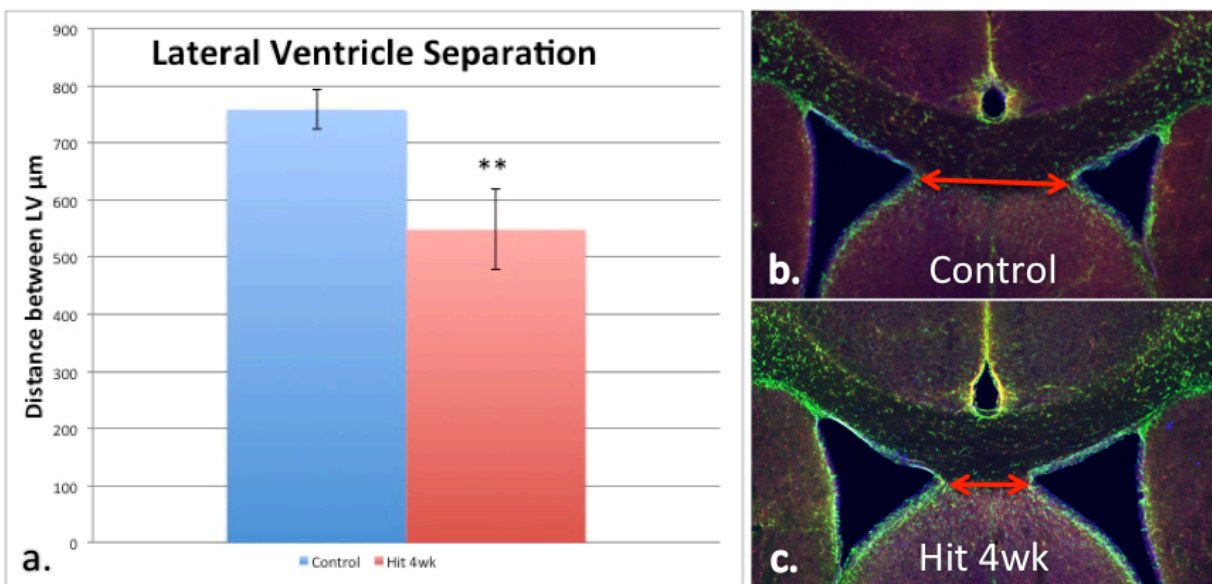


Figure 8. Decreased Lateral Ventricle Separation. a. Average distance between lateral ventricles is significantly decreased in C57 mice 4 weeks after rcTBI treatment compared to controls. b. Immunostained 10x magnification of control brain at periventricular region. c. Four-week hit brain shows decreased medial separation of the lateral ventricles along the corpus callosum.

## Can repetitive concussive traumatic brain injury (rcTBI) contribute to increased lateral ventricle volume?

The examination into the possibility of ventricular volume expansion in our mouse models in response to rcTBI was accomplished with the three-dimensional digital reconstruction protocol explained in depth in the *Materials and Methods* above.

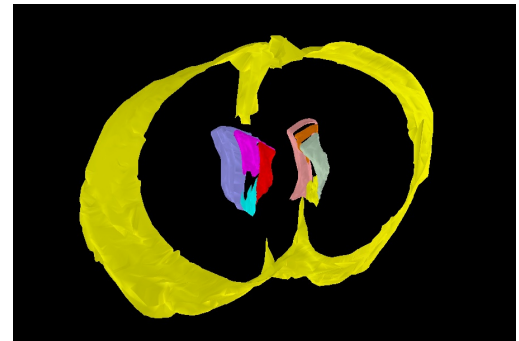


Figure 9. Three-dimensional rendering of lateral ventricle and whole brain created in NeuroLucida Explorer. .

Three-dimensional reconstruction from the most anterior portion of the lateral ventricles posteriorly to their connection with the third ventricle was performed on coronally sectioned brain tissue to obtain the ventricular volumes. Volume data was compiled from multiple time points for mice exposed to the concussive impacts and age-matched, isoflurane controls. We tested two strains of mice for ventriculomegaly in response to rcTBI: CD1 and C57B/6J. Our analysis revealed that due to the anatomical differences in the lateral ventricles, CD1 white mice had markedly inferior ventricle volumes compared to C57 mice and were an inappropriate model for ventricle volume measurements (Figure 10). Further analysis into volumetric enlargement of the lateral ventricles was conducted using C57 mice. Lateral ventricle volume was found to be significantly greater than age matched controls at the immediate, four week, and three month time points in the C57 mice (Figure 10). The data collected in the C57 mouse strain aligned with our original hypothesis: ventricle volume in the rcTBI-exposed mice would be increased compared to the controls at early time points after concussive impacts.

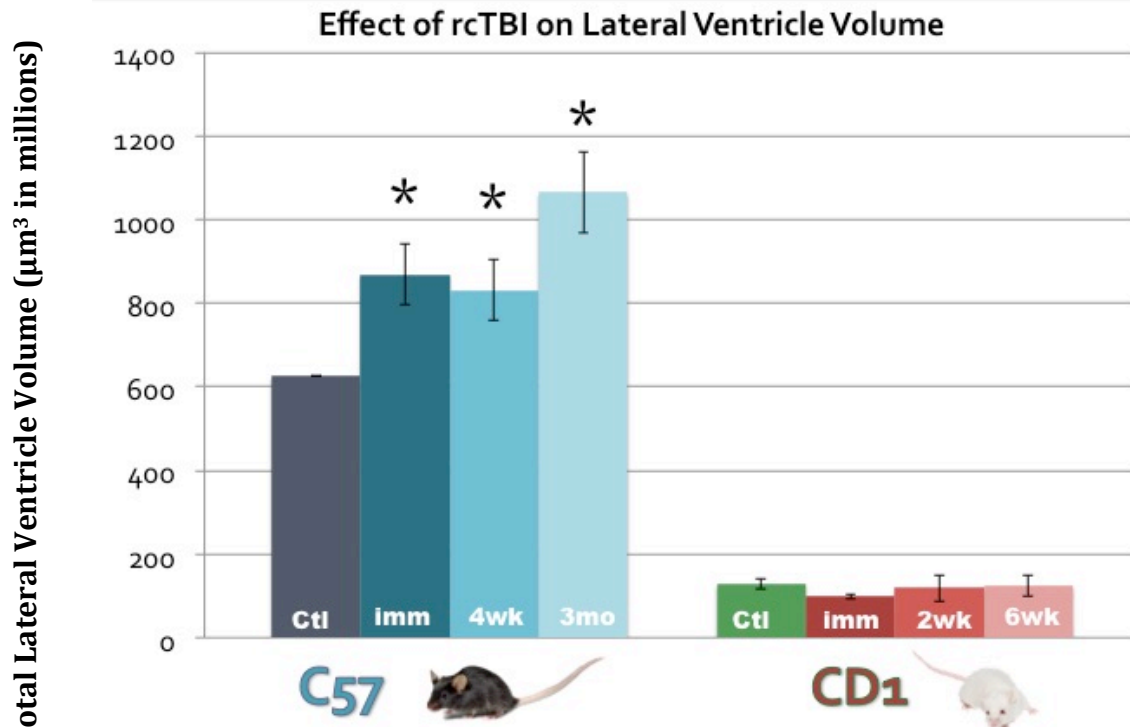


Figure 10. Lateral ventricular volume averages from each time point post-rcTBI compared to controls. C57 mouse ventricle volumes are significantly increased compared to controls after rcTBI. CD1 mice show inadequate lateral ventricle volumes for proper expansion analysis

The ventricle volume expansion did not correlate to any notable increase in whole brain volume compared to age-matched controls (data not shown).

## Discussion

We tested for astrocytic changes in the periventricular septal region by staining tissue with anti-GFAP fluorescent antibodies. The lack of GFAP upregulation in the cortex of rcTBI mice supports our claims that the weight drop model produces mild head impacts and does not cause cortical damage. Reactive astrocytes were found in the superior region of the septum with increased GFAP expression beginning immediately after rcTBI and intensifying through at least two weeks post-injury especially along the boundary of the corpus callosum.

The septum revealed significantly increased GFAP expression four weeks after rcTBI when above-threshold quantification techniques were applied. The periventricular septal region appears to have undergone not just general stress-related upregulation but area-specific damage response. The shearing forces at the septal boundaries are thought to cause neuronal and axonal damage that leads to leaking of intracellular molecules into the extracellular matrix. Intracellular constituents released by damaged cells act as chemoattractants for neuroglia and can lead to neuroinflammation involved in secondary injury of rcTBI. Reactive astrocytes have been identified to have a dual role in the neuroinflammatory pathway. Astrocytes were found to be beneficial to survival in the acute post-traumatic phase by releasing chemokines to recruit macrophages and monocytes.

Microglial immune response of the central nervous system was tested with IBA1 tissue staining and observation of microglial macrophage changes. Microglial IBA1 upregulation was observed at the earliest time point following rcTBI. Higher magnification imaging was used to identify aggregation of the microglial along the septal boundary with the corpus callosum. This focal aggregation of microglia suggests that the periventricular septum is specifically susceptible to damage from rcTBI. Imaging of individual cells in the region of aggregation found amoeboid-shaped morphologies consistent with activated microglia. As mentioned above, shearing forces in the septum are cause for the neuroinflammatory response and signaling of glial cells. Microglia undergo morphological changes and offer a similar dual role that may be important in recovery homeostasis. Activated microglia, through M1 or M2 subtypes, play important roles in the neuroinflammatory pathway. M1 microglia release pro-inflammatory molecules while M2 subtypes alternatively release anti-inflammatory molecules (Wang 2013). The two subtypes work in conjunction to control the inflammatory response to injury. Understanding the



interaction of M1 and M2 microglia in damage response proves important for discovering potential therapeutic interventions to prevent the development of CTE.

The appearance of both reactive astrocytes and activated microglia inferior to the corpus callosum in the septum may be attributed to novel findings of a bidirectional relationship between these glial cell types in cases of neurodegeneration. Reactive astrocytes have been shown to influence the recruitment of microglia to regions of injury through release of chemokines (Pekny 2014). Together, astrocytes and microglia create an early line of defense by surrounding damage lesions and protecting outlying healthy tissue. Over time this glial activation has been found to become inhibitory to CNS regeneration if the acute phase response is not resolved or worsens into glial scarring and accumulation of M1 pro-inflammatory microglia (Kumar 2012, Pekny 2014). Understanding the response timeline of astrocytes could provide an important opportunity for therapeutic intervention to avoid entering a phase of inhibitory action and possible progressive neurodegeneration as observed in CTE.

Our closed skull weight drop model was able to induce lateral ventricle volumetric expansion at early time points following injury. Three-dimensional ventricle reconstructions were developed to test for the presence of lateral ventricle volume expansion in two strains of mice: CD1 and C57B/6J. The lateral ventricles of the CD1 strain were observed to be markedly smaller than the C57 mice, which decreased the range of volumetric fluctuations. The smaller lateral ventricle volumes are due mainly to the increased presence of ventricular stenosis in CD1 mice, an adhesion of the lateral and medial ventricle walls that does not occur in humans. In the C57 black mouse strain, the degree of ventricle stenosis is often much less severe and has less effect on the overall ventricle volume. C57 mice displayed early promise in the capability of the ventricle reconstruction protocol.

Significant volumetric increase was found in response to rcTBI in C57 mice when compared to age-matched controls starting at the earliest time point after injury. We displayed that lateral ventricle enlargement can be induced with repeated mild, concussive head impacts in a mouse model. Our data correspond to characterization of early onset ventricular expansion in Stage 1 of CTE patients before the appearance of notable neuronal degeneration (Stein 2014). The development of such a model will allow for further examination into the causality and progression of ventricle expansion due to repeated head impacts. We postulate that the expansion of the ventricles can be attributed to cellular damage discovered in periventricular regions of the septum medial to the lateral ventricles. This hypothesis was supported with our findings of decreased septal width between lateral ventricles four-weeks post injury in C57 mice, correlated with the presence of reactive astrocytes and microglia in the region.

Though we are confident we are on the leading edge in the search for a clinically relevant mouse model, we can still articulate limitations and possible improvements of our study. The main limitation in our mouse model is the inability to observe pathological changes in a single individual over time. Current *in vivo* imaging techniques present inadequate quality for visualization of damage on the cellular level. Our model circumvents this limitation by sacrificing groups of concussed mice at multiple time points after exposure to investigate the pathological progression. In addition, age-matched controls are required for each round to understand the presumed resting state of the brain in the test subjects before rcTBI treatment.

Our current tissue imaging only surveyed up to six weeks post-injury, and in order to properly understand the pathological progression of such a long-term, degenerative disease we need to extend our experimental timeline. We predict that at later timelines we would find a

greater disparity in test subjects; with some subjects entering the progressive degeneration after prolonged neuroinflammatory response as predicated in CTE sufferers.

In order to better understand the effects of pathological damage to the septum, behavioral studies could be performed both before and at multiple time points after concussive impacts. Marked cellular and structural damage found in the brain gains true relevance when it can be correlated to clinical symptoms observed in CTE patients.

We hope that our findings will lead to an understanding of the pathological timeline of cellular response to rcTBI. A detailed progression of the brain's response to concussive impacts would help in developing strategies and treatments for head injury victims. Not all sufferers of rcTBI develop CTE and we believe there are underlining physiological factors that influence this observation. Understanding the physiology of CTE will offer insight into the role repeated head impacts have in the progression of neurodegeneration.

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